RECOMBINANT VENEZUELAN EQUINE ENCEPHALITIS VIRUS VACCINE

The present invention relates to a virus vaccine, specifically a
vaccine to Venezuelan equine encephalomyelitis virus (VEE), to
5   its preparation and pharmaceutically acceptable formulations and
methods of prophylactic and therapeutic methods of treatment
using said vaccine.

VEE virus is a mosquito-borne alphavirus which is an important
10   cause of epidemic disease in humans and of epizootics in horses,
donkeys and mules in certain parts of the world, in particular
the South Americas.

The existing VEE vaccine, TC-83, was initially produced by
15   attenuation of the Trinidad donkey strain (TRD) of VEE by
sequential passage in guinea pig heart cell cultures. However,
this vaccine is generally regarded as being inadequate for human
vaccination. This is mainly due to the high incidence of side
effects in vaccinees and the large proportion of vaccinees who
20   fail to develop neutralising antibodies (Monath et al. 1992,
Vaccine Research, 1, 55-68).

A vaccinia-based vaccine against VEE has been constructed
(Kinney et al. J. Gen. Virol. 1988, 69, 3005-3013). In this
25   recombinant, 26S RNA encoding structural genes of VEE were
inserted into the NYCBH strain of vaccinia. The recombinant
virus protected against sub-cutaneous challenge but had limited
efficacy against aerosol challenge with VEE.

30   The virulent Trinidad donkey strain of VEE and the attenuated
strain TC-83 have both been cloned and sequenced (R.M. Kinney et
al. Virology (1989) 170, 19-30) and the amino acid and
nucleotide numbering system used in this reference will be used
hereinafter. This work has revealed that there are a number of
35   amino acid changes between TRD and TC-83. The majority (five)
of these changes occur within the gene encoding the glycoprotein
E2.

The changes have been summarised as follows:

Table 1

change	Nucleotide		Amino acid	
Position	TRD	TC-83	TRD	TC-83
22, junction region	A	G	non-coding	
1053, E_2-7	G	U	Lys	Asn
1285, E_2-85	C	U	His	Tyr
1391, E_2-120	C	U	Thr	Arg
1607, E_2-192	U	A	Val	Asp
1866, E_2-278	U	C	none	
1919, E_2-296	C	U	Thr	Ile
2947, E_1-161	U	A	Leu	Ile
3099, E_1-211	A	U	none	
3874, 3'-non-coding region	UU	U	non-coding	

It has also been shown that the first 25 amino acids of the E2 glycoprotein represents a protective epitope. This region includes a single amino acid change (lys → asp) at amino acid 7 in the TC-83 construct as compared to the TRD strain. A 25bp synthetic peptide based on the TRD sequence VE2pep01(TRD), protected more mice from TRD virus challenge than a corresponding TC-83 based peptide (A.R. Hunt et al., Virology, 1990, 179, 701-711). More precise mapping of this epitope has been carried out (A.R Hunt et al., Vaccine 1995, **13**, 3, 281-288).

The applicants have found ways of increasing the protectiveness of a vaccine and in particular a vaccinia-based vaccine.

In particular, the applicants have found that the protectiveness of the vaccine may be increased either (a) by restoring the lysine residue at amino acid 7 of the E2 protein and/or (b) by modifying the promoter to increase expression of the protective construct.

Thus, in a first aspect, the present invention provides a vaccine for the therapeutic or prophylactic immunisation against Venezuelan Equine Encephalitis (VEE) virus, said vaccine

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comprising a vector which includes a sequence which encodes an
attenuated form of said virus which is capable of producing a
protective immune response, wherein the said sequence is such
that the amino acid at position 7 in the E2 protein of VEE is
5   lysine.

Suitably, the attenuated form of the VEE virus comprises a
derivative or variant of the TC-83 construct or an immunogenic
fragment thereof.
10   Other attenuated forms may be produced by the skilled person,
for example using known techniques such as serial passage
through another organism, or by recombinant DNA technology, for
instance by inactivating genes associated with the replication
or virulence of the virus. The structural gene encoding the E2
15   glycoprotein or a fragment encoding at least the N-terminal 19
amino acids should be retained in order to retain immunogenicity
of the construct.

Suitable fragments of the construct are those which include only
20   some of the structural genes of the VEE peptide or which encode
only part of the proteins encoded by said genes, provided the
construct encodes sufficient antigenic determinants to ensure
that it is capable of producing a protective immune response in
a mammal to whom the construct is administered.
25

As used herein, the term "variant" means that the construct
is different to the original strain but that it encodes
proteins and/or peptides which are the same or similar to
those of wild-type VEE or immunogenic fragments thereof.
30   Thus, the changes in the nucleotide sequence may be silent in
that they do not produce amino acid changes as compared to the
original strain, or they may produce amino acid changes
provided these do not alter function of the construct in
terms of its ability to produce a protective immune response
35   against VEE. For example, the construct may encode peptides
or proteins which are 60% homologous to the wild-type proteins
or peptides, suitably more than 80% homologous and preferably
more than 90% homologous to the native protein sequence, and
provided they produce antibodies which are cross-reactive with
40   wild-type VEE, the protective effects of the construct may be
retained.

"Derivatives" may have broadly similar structures but they
are derived by manipulating the original constructs using
recombinant DNA technology or chemical modification if
appropriate.

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The vector may contain the usual expression control functions
such as promoters, enhancers and signal sequences, as well as a
selection marker in order to allow detection of successful
transformants. The selection of these will depend upon the
10   precise nature of the vector chosen and will be known to or
readily determinable by a person skilled in the art.

Suitably the vector is a viral vector, for example a vector
derived from vaccinia, adenovirus, or herpes simplex virus (HSV)
15   BCG or BCC. It is suitably attenuated itself, to minimise any
harmful effects associated with the virus on the host.

Preferably, the vector is derived from vaccinia virus, as it has
many properties which make it a suitable vector for vaccination,
20   including its ability to efficiently stimulate humoral as well
as cell-mediated immune responses. Vaccinia has proven utility
as a vaccine vehicle, following the Smallpox eradication
programmes. It provides the potential for multi-valent
vaccine construction and for oral administration. There are
25   many attenuated strains currently available.

A suitable selection marker for inclusion in a vaccinia vector
is the gpt marker gene.

30   A VEE vaccine was constructed using a WR strain of vaccinia in
this work. Preferably, a more highly attenuated strain of
vaccinia which would be more acceptable for use in humans is
employed. Such strains include Lister, which was used for wide
scale vaccination against smallpox, NYVAC (Tartaglia et al,
35   (1992). AIDS Research and Human Retroviruses 8,1445-1447) which
contains specific genome deletions, or MVA (Mayr et al, (1975)
Infection 3, 6-14) which is also highly attenuated.

Vaccines based upon viral vectors are suitably formulated for
40   parenteral administration as described above. However, it is
possible to formulate such vaccines for oral administration, for

example by incorporating the vector into a gut-colonising
microorganism such as *Salmonella* and particularly *S.
typhimurium*.

5   pTC-5A is a plasmid clone of cDNA encoding the structural genes of VEE
     virus strain TC-83 (Kinney et al. J. Gen. Virol. (1988) **69**, 3005-
     30130). The VEE cDNA is situated downstream of the vaccinia 7.5K
     promoter which drives expression of the VEE structural proteins when
     the plasmid is used to construct recombinant vaccinia viruses.
10   Modified 7.5K vaccinia promoters have previously been prepared
     (Davison & Moss, J. Mol. Biol. **210**, (1989) 749-769). It has been
     found that certain substitution mutations increase the strength of the
     promoter. By using synthetic promoters which include substitution
     mutations, the amount of VEE proteins produced from the recombinant
15   virus was increased.

     Thus in a further aspect of the invention, there is provided a
     vaccine for the therapeutic or prophylactic immunisation
     against Venezuelan Equine Encephalitis (VEE) virus, said vaccine
20   comprising a vaccinia virus vector which encodes an attenuated
     form of the VEE virus or a variant or fragment thereof which is
     capable of producing a protective immune response against VEE
     virus, expression of the said attenuated VEE virus being under
     the control of a synthetic 7.5K vaccinia promoter which has been
25   subject to mutation which increases the level of VEE virus
     protein production as compared to the wild-type 7.5K promoter.

     In particular, it has been found that substitution mutations
     within the 7.5Kd promoter can be effective. These may be
30   illustrated by the following Table:

     Wild-type 7.5K promoter:

          TAAAAGTAGAAAATATATTCTAATTTATTGCAC   (SEQ ID No 1)
35
     Substitution Mutations (emboldened)
          TAAAAATTGAAAATACATTCTAATTTATTGCAC   (SEQ ID No 2)

          TAAAAATTGAAAATATATTCTAATTTATTGCAC   (SEQ ID No 3)
40
     Inclusion of a synthetic 7.5K vaccinia promoter in WR103 has

been found to increase expression of the downstream VEE cDNA, leading to a 3.59-fold increase in protein production.

The vaccine may comprise the vector itself but it is suitably formulated as a pharmaceutical composition in combination with a pharmaceutically acceptable carrier or excipient. Such compositions form a further aspect of the invention. The compositions may be in a form suitable for oral or parenteral application.

Suitable carriers are well known in the art and include solid and liquid diluents, for example, water, saline or aqueous ethanol. The liquid carrier is suitably sterile and pyrogen free.

The compositions may be in the form of liquids suitable for infusion or injection, or syrups, suspensions or solutions, as well as solid forms such as capsules, tablets, or reconstitutable powders.

Constructs for use in the vaccines of the invention may be prepared by various means as will be understood in the art, ranging from modification of available constructs such as the wild-type virus using recombinant DNA technology or by synthetic means. Recombinant DNA techniques include site directed mutagenesis, optionally involving PCR amplification as illustrated hereinafter.

As illustrated hereinafter, recombinant vaccinia virus was constructed which expressed the structural genes of VEE as produced by a modified form of TC-83. The ability of the recombinant virus to elicit protective immune responses against virulent VEE disease was investigated.

In yet another embodiment, the vaccine further comprises a cytokine or an active fragment or variant thereof. The cytokine may itself be incorporated into the vaccine formulation, or more suitably, the vector may include a coding sequence which means that the cytokine is co-expressed by the vector. Examples of suitable cytokines include interleukin 2 (IL-2) and interleukin 6 (IL-6).

A particularly suitable cytokine is interleukin 2 (IL-2), which
may be expressed from for example a vaccinia virus recombinant.
IL-2 is known to be responsible for the clonal expansion of
5   antigen-activated T cells (Smith, (1984) *Reviews in Immunology*
2, 319-333).

Alternatively, antibody levels can be enhanced using other
cytokines. For example, expression of IL-6 by vaccinia vectors
10 has been shown to induce a high level of IgG_1 (Ruby *et al*, 1992
Vaccine Research 1, (4), 347-356), and IL-5 and IL-6 induced
mucosal IgA responses to co-expressed influenza HA (Ramsay *et
al*, (1994) Reproduction, Fertility and Development 6, 389-392).

15 The vaccine of the present invention may be used to treat humans
or animals. In particular it may be given to horses, as a
veterinary vaccine, to prevent infection, or as a prophylactic
or therapeutic vaccine for humans.

20 The vaccine of the invention may be incorporated into a
multivalent vaccine in order to increase the benefit-to-risk
ratio of vaccination.

The dosage of the vaccines of the invention will depend upon the
25 nature of the mammal being immunised as well as the precise
nature and form of the vaccine. This will be determined by the
clinician responsible. However in general, when using a virus
vector such as a vaccinia virus vectors, dosages of the vector
may be in the range of from 10^4-10^{12}pfu (pfu = particle forming
30 units).

The vaccines of the invention will produce an immune response in
test animals including the production of antibodies. These
antibodies may be useful in passive vaccination programmes or in
35 diagnosis of VEE virus disease. For diagnostic purposes, the
antibodies may form part of a kit as is conventional in the art.

The invention will now be illustrated by way of Example with
reference to the accompanying drawings in which
40
Figure 1 shows the construction of chimeric plasmids used for

generation of recombinant vaccinia viruses;

Figure 2 shows the results of a immunofluoresence assay using
polyclonal antiserum to TC-80;

5

Figure 3 is a graph showing the results of an experiment to
quantify by ELISA the amount of VEE protein expressed by
strains; and

10   Figure 4 is a graph showing the results of an experiment to find
the level of anti-VEE IgG in animals vaccinated with various
strains of the invention.

In the Examples, relative protein levels were calculated from
15   ELISA data using regression analysis performed by Minitab
statistical analysis software (Minitab Inc., State College,
PA, USA). Serum antibody levels were compared by the two
sample t test. Contingency tables were analysed by Fisher's
exact test. P values of <0.05 were taken to be significant.
20

Example 1
Alteration of the E2 protein sequence
pTC-5A, a plasmid clone of cDNA encoding the structural genes
of Venezuelan Equine Encephalitis virus, strain TC-83 was
25   obtained from Dr. R, Kinney (Kinney et al, 1988, Journal of
General Virology 69, 3005-3103). An Eco RI fragment
containing the VEE cDNA was removed from pTC-5A and inserted
into p1113 (Carroll, 1993, Ph.D. thesis, Faculty of Medicine,
University of Manchester; Fig 1a) which is a shuttle vector
30   used for insertion of genes into the thymidine kinase locus of
vaccinia with dominant selection of recombinant viruses based
on resistance to mycophenolic acid (Falkner & Moss, 1988,
Journal of Virology 62, 1849-1854). The resulting plasmid,
pAB100, was mixed with Lipofectin ™ (Life Technologies) and
35   used to transfect CV-1 cells infected with vaccinia virus,
strain WR. Recombinant viruses were designated WR100 and were
subjected to three rounds of plaque-purification before
preparation of stocks as described earlier (Mackett et al,
1985 DNA cloning (Volume II): a practical approach).
40

The sequence of VEE E2, strain TC-83, situated in pTC-5A, was

altered by one nucleotide substitution from T to G as position
1053 as compared to wild-type VEE TRD (Johnson et al. J. Gen.
Virol. 1986, 67, 1951-1960). This resulted in an amino acid
change from asparagine to lysine in the E2 protein when
5   expressed from the vaccinia virus.

In order to perform this particular amino acid change, the
following manipulations were carried out..

10   A cleavage site for restriction enzyme Nsi I occurs close to the
site of the required nucleotide substitution. A second Nsi I
site is situated about 500bp upstream. Oligonucleotide primers
were used to amplify the DNA sequence between the Nsi I sites
using the Polymerase Chain Reaction (PCR). The downstream
15   primer contained a nucleotide mismatch corresponding to the TRD
sequence at this point.

The primer sequences are listed below. The Nsi I cleavage sites
and the position of the substituted nucleotide are underlined.
20

Primer 1 designated " Nsi 1"

       5'   GCC GAT GCA TGT GGA AGG C   3'

25   Primer 2 designated " Nsi 2"

       5'   ATC TGA TGC ATC TGG CCA TGT AAG GGC GCG TTA GCT TAT
       ACT CCT TAA ACA GC   3'

30   The PCR product was digested with Nsi I and used to replace the
corresponding Nsi I fragment in pTC-5A, generating plasmid
pAB101. The nucleotide sequence of the relevant region in
pAB101 was obtained to verify the sequence alteration.

35   pAB101 was then digested with Eco R1 to remove the VEE 26S RNA
coding sequence which was transferred to the vaccinia shuttle
vector plasmid p1113. P1113 contains the selectable marker gpt
which allows selection of recombinant vaccinia viruses. The
plasmid constructed by the addition of the VEE sequence to p1113
40   was designated pAB102.

Example 2

Substitution of 7.5K promoter for a synthetic promoter in pAB102

A synthetic 7.5K vaccinia promoter was designed, based upon work by Davison and Moss (supra.). Complementary oligonucleotides

5   were designed with 5' *Bam* HI and 3' *Eco* RI ends. The oligonucleotides were annealed and ligated into the plasmid pT7Blue (available from AMS Biotechnology (UK) Ltd). The plasmid clone was digested with *Bam* HI and *Eco* RI and the DNA fragment containing the synthetic promoter was isolated and cloned into

10 the plasmid pAB102 which had been cut with the same enzymes. This resulted in the generation of plasmid pAB103 (Figure 1) which contains the synthetic promoter upstream of the VEE 26S RNA coding sequence. Vaccinia WR strain was transformed with PAB103 to produce the recombinant vaccinia virus WR103.

15

The sequence of the oligonucleotides used is given below. Substitutions in the 7.5K promoter sequence are given in bold type. Insertions are underlined. Oligonucleotide " tails" containing restriction enzyme cleavage sites are italicised.

20

Oligo 1 designated " 7.5KF2"

5'   *ACG CGG ATC* C<u>AA AAA TTG AAA AAC TAG C</u>TT AAA AAT TGA AAA ACT ATT CTA ATT TAT TGC ACG *AAT TCC G*   3'

25 ### Oligo 2 designated " 7.5KR2"

This is the reverse complement of 7.5KF2.

The amount of VEE proteins produced by the recombinant virus WR103 was measured using enzyme linked immunoabsorbant assay

30 (ELISA).

Example 3

Analysis of protein expression

35 VEE viral proteins were visualised by indirect immunofluorescence of infected CV-1 cells. CV-1 monolayers (25cm^2) were infected with virus at a multiplicity of 2 p.f.u. per cell. At 24 hours post infection, cells were scraped into the growth media and washed once with phophate-buffered saline

40 (PBS) containing 0.1% bovine serum albumin. Cells were spotted onto slides, air-dried and fixed in acetone. Binding

of mouse polyclonal antiserum raised against VEE strain TC-80
(provided by Dr. A.D.T. Barrett, University of Texas) was
detected with fluorescein isothiocyanate-conjugated goat anti-
mouse IgG (Amersham International plc).

5

Examination of cells infected with WR100 or WR103 showed that
WR103-infected cells fluoresced more brightly than the WR100-
infected cells (Figure 2).

10   Quantification of VEE viral protein expression was carried out
using an enzyme-linked immunosorbent assay (ELISA). CV-1
monolayers (150 cm^2) were infected with virus at a multiplicity
of 10 p.f.u. per cell and harvested at 24 hours post infection
by scraping into the growth media. Cells were washed once in
15   PBS and resuspended in T9 buffer (10 mM Tris.HCl; 1 mM EDTA;
pH 9.0). Samples were frozen, thawed and sonicated for 1
minute in a sonicating bath. Cells debris was pelleted for 5
minutes at 1800 g and the supernatant was centrifuged for 30
minutes at 10,000 x g. The supernatant was removed and stored
20   at -70°C. The cell lysate preparation was diluted 1/30 in
bicarbonate buffer (Sigma), 100 l volumes were added to wells
of a microtitre plate and the antigen was allowed to bind at
37°C for 1 hour. Lysates were replaced with 200 l/well of
saline containing 10% formaldehyde. Plates were incubated at
25   room temperature for 20 minutes, then washed 6 times with PBS
containing 0.1% Tween (PBST). Mouse polyclonal anti-TC80 was
serially diluted in blocking solution (0.5% dried milk/PBST),
added to wells, and the plates were incubated for 1 hour at
37°C. Plates were washed 3 times in PBST before addition of
30   horseradish peroxidase-conjugated mouse specific antibody
(diluted 1:1000 in blocking solution) and incubated for 1
hour at 37°C. Plates were washed 3 times before addition of
ABTS in citrate buffer and incubation at room temperature for
1 hour. Colour development was measured at A_{414}.

35

This quantification process revealed that WR103-infected cells
contained 3.59-fold more VEE protein than WR100-infected cells
(Figure 3).

40   Quantification of vaccinia protein in these samples had
demonstrated equivalent amounts in each (data not shown), so

it must be assumed that the difference in VEE protein content
is due to different expression levels of the encoded VEE cDNA.

5   **Example 4**
     <u>Protective effect of Vaccinia recombinants</u>
     Groups (10) of female 6-8 week old Balb/c mice were inoculated
     with PBS or with 10^8 p.f.u. of vaccinia viruses by intra-
     muscular injection, or with 10^5 p.f.u. of TC-83 by sub-
10   cutaneous injection. Serum was taken for measurement of
     immunoglobulins to VEE proteins.

     The vaccinated mice were challenged with two different doses of
     virulent VEE strain TRD at 35 days after immunisation. The
15   survival rates after 14 days are presented in Table 2.

                              Table 2

Strain	10pfu TRD	100 pfu TRD
WR	0/10	0/10
WR100	1/10	2/10
WR103	6/10	6/10
No treatment	0/10	0/10

20   WR100: Vaccinia/VEE recombinant
     WR103: Vaccinia/VEE recombinant produced in Example 2 above.

     These results show that genetic manipulation of the recombinant
     virus has improved the protection afforded by the construct. A
25   significant improvement in protection of mice following sub-
     cutaneous challenge with TrD was seen when WR103 was used for
     vaccination, compared with WR100 ($P<0.05$, Table 2). WR100
     protected up to 20% of mice whereas WR103 protected 60% of
     mice. There was not a significant difference between numbers
30   of mice protected when challenge doses of 10 p.f.u. or 100
     p.f.u. of TrD were used. The challenge dose had previously
     been titrated to show that 1 p.f.u. of TrD approximates to 2-3
     LD_{50} doses (data not shown).

Example 5

Immunoassays

VEE virus-specific immunoglobulin in serum was measured by
enzyme-linked immunoassay as follows. Wells of a microtitre
5   plate were coated with purified TC-83 at 37°C for 1 hour.
Serum was diluted serially in blocking solution and allowed to
bind to antigen-coated wells overnight at 4°C. Plates were
washed 3 times and incubated with horseradish peroxidase-
conjugated anti-mouse immunoglobulin at 37°C for 1 hour.
10   Plates were washed and incubated with TMB substrate for 20
minutes before measurement of colour development at A_{450}.

All vaccinia-inoculated mice responded to the vaccination by
the detection of immunoglobulin to vaccinia virus in serum
15   (data not shown). Immunoassay to measure TC-83 antibody
failed to detect anti-VEE IgG in WR100 samples. WR103 samples
contained a detectable level of anti-VEE antibody although
this was substantially lower than the amount found in serum
from mice vaccinated with TC-83 (Figure 4).
20

Neutralising antibody was measured by a plaque reduction test.
Serum (10µl) was incubated with TC-83 (50µl) and maintenance
medium (140 µl) for 1 hour at room temperature. Maintenance
medium (800µl) was added and the suspension was used to infect
25   confluent monolayers of BHK-21 cells grown in 6-well plates.
Plates were incubated at 37°C for 3 days. A 50% reduction in
the number of plaques per well, compared to control wells, was
indicative of the presence of neutralising antibody.

30   Neutralising antibody to TC-83 was found in serum from mice
vaccinated with TC-83 but was not detected in serum from mice
vaccinated with WR100 or WR103 (data not shown). Although
neutralising antibody is usually found in mice which are
protected against VEE challenge, protection has previously
35   been reported in the absence of detectable neutralising
antibody (Kinney et al, 1988a, Journal of Virology 62, 4697-
4702).

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